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USE OF FLUORESCENT MATERIALS FOR THE INDIRECT DETECTION OF ANTIBIOTICS IN MILK

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SUMMARY

The addition of a combination of fat-soluble fluorescein (Fluoral) and uranine, as a "marker," to penicillin preparations intended for intramammary infusion, was found to provide a rapid and satisfactory means for detecting the antibiotic in milk. The marker was detected visually in the milk for 48 hr. after treatment and with ultraviolet light for 96 hr. The marker was nontoxic to the treated animals and did not affect milk production. Statistical analysis of the data showed a close correlation between the excretion of marker and penicillin from the treated udders.

A rapid, sensitive, and reliable means for detecting antibiotics in milk is needed urgently by dairy manufacturers, dairy farmers, and public health officials. Although several sensitive and apparently reliable tests for antibiotics have been proposed recently, all of them are laboratory tests that require from 2½ to 8 hr. *(1, 3, 4)*. The time, laboratory equipment, and skill required for these tests reduce their usefulness, particularly to cheese-makers and farmers.

A rapid and practical test for antibiotics in milk would enable farmers to determine definitely how long to withhold the milk from antibiotic-treated cows; for manufacturers to avoid serious economic loss resulting from the injurious effects of antibiotics on bacterial starters used in making cheese and fermented milks, and for public health officials to make routine tests of large numbers of milk samples.

The incidence and concentrations of antibiotics in market milk have been reported recently *(2, 5, 6)*, and cheese-makers have frequently encountered complete failure of bacterial starters, owing to the presence of antibiotics in the milk.

In 1950, the Agricultural Research Service encountered sporadic appearances of antibiotics in the milk being used for making experimental cheese. At that time, the feasibility of incorporating a dye in all antibiotic preparations to be used for intramammary infusion was investigated. Such a dye would serve as a visual index of antibiotic contamination in milk. The ideal dye for this purpose would have to remain in the milk as long as the antibiotic, have no effect on the antibiotic activity, and be nontoxic and nonirritating to the cows' udders. Although some of the dyes tested in the early studies were readily detectable visually for 48 hr. after their infusion into udders, they failed to remain in the milk as long as did penicillin.

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Recently, these studies have been resumed, using fluorescent materials and additional dyes as antibiotic markers. Although several phases of the work remain to be completed, preliminary experiments indicate that it is feasible to add such markers to antibiotics intended for veterinary use. This report briefly presents the pertinent findings.

EXPERIMENTAL PROCEDURE

Various chemicals were screened, to find an agent that could be used as a tracer, or marker. Selection was based primarily upon fluorescence, color, and odor. The list of chemicals tested included certified food colors, cosmetic dyes, food-flavoring compounds such as vanillin, cinnamaldehyde, and methyl anthranilate, odoriferous materials such as furfuryl mercaptan and gamma undecalactone, and various fluorescing agents such as the fluoresceins, chlorophylls, and hydroxycoumarins. In the preliminary study, each material was tested for ease and limits of detection in whole milk. Milks containing fluorescent materials were diluted serially and measured for fluorescence with a 2-amp. long-wave ultraviolet (3660Å) lamp. Effects of the chemical upon the antibiotic activity of penicillin were determined by penicillin assays with *Streptococcus thermophilus*. Materials that appeared to have value as tracers were tested further, for their possible effects on antibiotic activity during storage. In most instances, mixtures of the chemical and penicillin in mineral oil and also in a water-in-oil preparation (Penicle),[2, 3] were stored at room temperature. The limits of detection for tracer agents were established visually by a number of individuals, both under normal and under ultraviolet light. Agents selected on the basis of the in vitro tests were subjected to further tests, by injection into udders, either alone or in combination with penicillin.

Three separate trials were conducted to evaluate the marker materials in the in vivo studies. Some markers were subjected to additional trials if further testing seemed advisable. Each trial was designed as a balanced incomplete block experiment, with four marker treatments assigned at random to the four quarters of the udder of each of six cows representing a wide range in milk production. A common source of oil-penicillin preparation was used in preparing the emulsions of tracer materials. In every instance, one 15-ml. dose, containing known amounts of marker, oil, and penicillin, was administered. The dose of penicillin ranged from 100,000 to 300,000 units, and the dose of tracer from 250 to 400 mg. Quarter-milkers were used to collect samples of milk from the individual quarters for at least 96 hr. after infusion. Quarter-milkings prior to treatment were used as controls for pH measurements and leucocyte counts. Close veterinary scrutiny and complete milk production records were maintained. Milk samples from the treated quarters were tested for leucocytes, pH, penicillin content, and for the presence of marker. Further, milks containing marker

[2] Pitman and Moore.

[3] The use of trade names is for the purpose of identification only, and does not imply endorsement of the product or its manufacturer by the U. S. Department of Agriculture.

were diluted with normal herd milk to determine the extent of dilution without loss of marker detection.

Trial I. 4-Methyl umbelliferone, methyl anthranilate, esculin, and uranine (sodium fluorescein) were used as marker materials. Each test dose contained 250 mg. of marker and 100,000 units of penicillin G in 15 ml. of an oil base containing equal parts of light mineral oil and petrolatum.

Trial II. Uranine, oil-soluble fluorescein (Fluoral 7 Ga.)[3, 4] a combination of uranine and oil-soluble fluorescein, and spirit-soluble fluorescein were compared as marker materials. Each dose contained 250 mg. of marker and 100,000 units of penicillin in an oil base. The oil base in this trial was Penicle.

Trial III. Esculin, a combination of esculin and Amaranth Red No. 2, oil-soluble fluorescein, and oil-soluble chlorophyll were tested. Each test dose contained 400 mg. of marker and 300,000 units of penicillin G in 15 ml. of Penicle.

Data accumulated from these trials were statistically analyzed to evaluate the marker materials, as well as to determine the effect of the level of milk production on the rate of excretion of the antibiotic and marker from the udder.

RESULTS

The dyes tested to date do not seem to have as much potential as tracer materials as do the fluorescing agents. For the most part, the odorous or flavored materials were unsatisfactory as tracer agents. The limits of identification for these materials varied tremendously from one individual to another, and to a lesser degree with milk quality. In this report, only the marker materials that have been tested by udder infusion will be considered.

Limiting concentrations of marker material at which color of fluorescence was apparent in milk are shown (Table 1). Laboratory attempts to isolate tracer materials from the milks were successful with a few agents, but such procedures were not considered to be practical for routine identification. The fluorescein derivatives could be detected in milk both by color and by fluorescence in rather high dilution. As may be noted, esculin and 4-methyl umbelliferone were detected in milk only by the use of ultraviolet light. Methyl anthranilate was detected by ultraviolet light and also by its grape-like odor and taste.

None of the markers caused a degradation in the antibiotic activity of penicillin stored in mineral oil at room temperature over a period of 4 mo. In the

[4] General Dyestuff Company.

TABLE 1

Limiting concentration of marker detected in whole milk

Marker	Ordinary light	Ultraviolet light
	(p.p.m)	
Esculin	Not detectable	1
Uranine	200	10
Oil-soluble fluorescein	200	10
Spirit-soluble fluorescein	100	10
4-Methyl umbelliferone	Not detectable	1
Methyl anthranilate	Not detectable	10
Amaranth Red No. 2	100	Nonfluorescent
Oil-soluble chlorophyll	200	200

Penicle preparation stored at room temperature, Amaranth Red caused a marked reduction in penicillin activity. The limits of identification for the markers in milk were not affected by storage with penicillin in any of the oil preparations.

In the in vivo studies, it became evident that some of the materials were unacceptable as tracer agents, whereas others showed considerable promise. Some agents appeared to be slightly toxic and others were absorbed from the udder. Marker agents were evaluated after a statistical analysis of the data from the penicillin and marker determinations on the treated milks from individual cows. The values listed (Table 2) are the averages for the six cows used to test each marker. These data show the number of milkings after treatment during which penicillin was being shed in the milk at different levels, and the number of milkings during which the markers could be detected at various dilutions. In every instance, penicillin was detected at the 72-hr. period and, in many instances, at 96 hr. or longer. This emphasizes the need for a test to detect antibiotics in milks as long as they are excreted, rather than depending on a fixed period of 72 hr. for the milk to be withheld.

Trial I. The persistency of the marker materials in the udder did not appear statistically different. However, esculin and uranine were definitely superior with respect to detectability in the treated milks. The veterinary report indicated clearly that 4-methyl umbelliferone and methyl anthranilate were unsuitable as marker agents. 4-Methyl umbelliferone caused a slight but definite irritation in all the test quarters, whereas methyl anthranilate, although nontoxic, partially was absorbed in the udder and appeared in the urine of the cows. No rise in leucocyte count or pH of the milk was noted either with esculin or with uranine.

Trial II. In this trial, the marker persistency at the zero and 1 : 4 dilutions showed a highly significant difference between markers. Oil-soluble fluorescein had the greatest persistency and was readily detected in the herd milk. The leucocyte count and pH of treated milks remained constant.

Trial III. In Trial III, marker persistency at the zero and 1:4 dilutions showed highly significant differences between markers. However, the differences between esculin, a combination of esculin and Amaranth Red, and oil-soluble fluorescein were not significant. Chlorophyll was definitely inferior to the three other test materials. (Results of recent tests with increased doses of chlorophyll suggest that it has much greater value as a marker than is indicated in Table 2.)

The combination of uranine and oil-soluble fluorescein was very effective as a marker, in that it definitely colored the milk for at least 48 hr. after infusion, and was detectable by ultraviolet light for 96 hr. after infusion.

The retention of penicillin and marker in the quarter was influenced greatly by the quantity of milk being produced. Low producers retained penicillin and marker in the quarter as much as 24 to 48 hr. longer, and at higher levels, than did the higher producers.

Increasing the penicillin dose from 100,000 to 300,000 units had little effect upon the number of milkings in which penicillin persisted. However, a greater proportion of penicillin appeared in the earlier milkings with the larger dose.

TABLE 2

Persistence of penicillin and markers in milk

Trial	Marker	Marker dose	Penicillin dose	Av. milk yield per quarter	Penicillin detected at levels of: 0.1	0.2 (units/ml)	1.0	Marker detected at dilutions of: 0	1–4	1–9
		(mg.)	*(units)*	*(lb.)*	*(Av. No. milkings)*					
I	1. 4-Methyl umbelliferone	250	100,000	......	6.25	5.34	3.22	2.05	0.55	0.02
	2. Methylanthranilate	250	100,000	......	7.79	6.71	4.18	1.31	0.19	0
	3. Esculin	250	100,000	......	7.01	6.06	3.86	9.10	5.88	3.77
	4. Uranine	250	100,000	......	7.17	5.88	3.22	10.24	6.03	3.35
II	1. Uranine	250	100,000	3.01	8.24	7.24	4.90	2.73	1.58	0.92
	2. Oil-soluble fluorescein	250	100,000	3.41	7.34	6.50	4.37	8.70	5.36	3.16
	3. Combination uranine and oil-soluble fluorescein	125 (each)	100,000	4.00	8.00	7.06	4.73	5.51	3.73	2.55
	4. Spirit-soluble fluorescein	250	100,000	3.92	7.44	6.54	4.45	4.57	3.15	2.20
III	1. Esculin	400	300,000	3.88	7.15	6.45	4.81	7.69	5.96	4.81
	2. Combination, Amaranth Red and esculin	400 (each)	300,000	3.71	7.24	6.50	4.77	7.54	5.88	4.78
	3. Oil-soluble fluorescein	400	300,000	3.71	7.18	6.48	4.84	7.96	5.47	3.82
	4. Oil-soluble chlorophyll	400	300,000	3.78	8.03	7.21	5.30	2.94	0	0

Increasing the marker doses had a similar effect, especially with the water-soluble agents. The oil-soluble materials appeared to remain in the udder slightly longer and at a higher concentration level than did most of the water-soluble agents.

Table 3 shows the correlation coefficients between penicillin and marker persistency. The correlation was obtained for the over-all milkings as well as for the within-milkings. It is of interest to note that a combination of uranine and oil-soluble fluorescein was more closely associated with penicillin persistency than any other marker, or combination of markers.

In Trial II, an increase of 1 lb. of milk yield decreased penicillin persistency (at a level of 0.10 penicillin unit) by 0.28 milking. In Trial III, an increase of 1 lb. of milk yield decreased penicillin persistency by 0.115 milking. The fact that milk yield had less effect on penicillin persistency in Trial III might be attributed to the fact that dosage of penicillin administered in Trial III was much greater than in Trial II.

In Trial II, the statistical analysis of data showed that an increase in 1 lb. of milk yield decreased marker persistency at the 1 : 4 dilution level by 0.31 milking. In Trial III, this decrease was 0.145 milking. Again, the dosage of the marker was greater in Trial III than in Trial II. It is of interest to note the strong parallel in the influence of milk yield on the persistence both of penicillin and of marker.

TABLE 3

Correlation coefficients between penicillin and marker persistency

Trial	Marker	Milkings Total	Milkings Within
I	4-Methyl umbelliferone	0.67	0.07
	Methyl anthranilate	0.22	−0.10
	Esculin	0.91	0.84
	Uranine	0.90	0.85
II	Uranine	0.75	0.45
	Oil-soluble fluorescein	0.81	0.61
	Combination of oil-soluble fluorescein and uranine	0.94	0.82 [a]
	Spirit-soluble fluorescein	0.85	0.22
III	Esculin	0.88	0.26
	Combination of Amaranth Red and esculin	0.86	0.20
	Oil-soluble fluorescein	0.88	0.34
	Oil-soluble chlorophyll	0.31	−0.19

[a] Perhaps this indicates that a combination of 250 mg. of oil-soluble fluorescein and 125 mg. of uranine is most closely associated with penicillin persistency.

DISCUSSION

Hydroxycoumarin derivatives such as esculin, umbelliferone, and 4-methyl umbelliferone are known for their fluorescence in ultraviolet light. Small quantities were easily detected in milk by their fluorescence. Esculin has been reported to be detectable in water at a concentration of 0.1 p.p.m., and we found it to be strongly fluorescent in milk.

The optimal pH for fluorescence was considered for all materials. The fluorescence of 4-methyl unbelliferone in milk was increased tenfold by adjusting the milk pH to 9.0. The brilliant blue fluorescence of esculin was more difficult to detect in old milk, as there was a tendency for the milk, as it aged, to change from a greenish to a blue fluorescence. Methyl anthranilate was selected as a possible tracer agent, because it can be added to foods, has a strong grape-like aroma and flavor and, in addition, is fluorescent in ultraviolet light. Although methyl anthranilate and 4-methyl umbelliferone could be extracted in *n*-butyl alcohol, they proved to be unsatisfactory in other respects in the in vivo studies.

Plant chlorophyll is known to produce a fiery-red fluorescence and, as it has been reported to have some medicinal properties, it seemed an excellent material to include in the study. Of the chlorophyll preparations tested, the oil-soluble form showed more potential as a marker. Small quantities in milk could be detected by extraction in *n*-butyl alcohol.

Fluorescein derivatives, as might be expected, gave a bright-green fluorescence and color to the milk. Some difficulty may be encountered in differentiating the yellow fluorescence of uranine from that of normal milk in concentrations of 1 p.p.m. or less. However, adjustment of the milk to pH 7.5 to 8.0 aids in the detection of uranine by diminishing the normal fluorescence of milk. Oil-soluble fluorescein appeared in the treated milks attached to the fat globule. The floating fluorescent globules were easily detected in the fluid milk by ultraviolet light.

In our studies, no evidence of toxicity was noted when esculin, chlorophyll, uranine, and oil-soluble fluorescein were injected into the udders of cows. A lack of toxic effects of these agents when ingested by animals has not been determined in this study. The cost of marker material is an important factor in considering it for commercial use. The amount of oil-soluble fluorescein and uranine added to the veterinary antibiotic preparation should not increase the cost more than one-half cent per dose.

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